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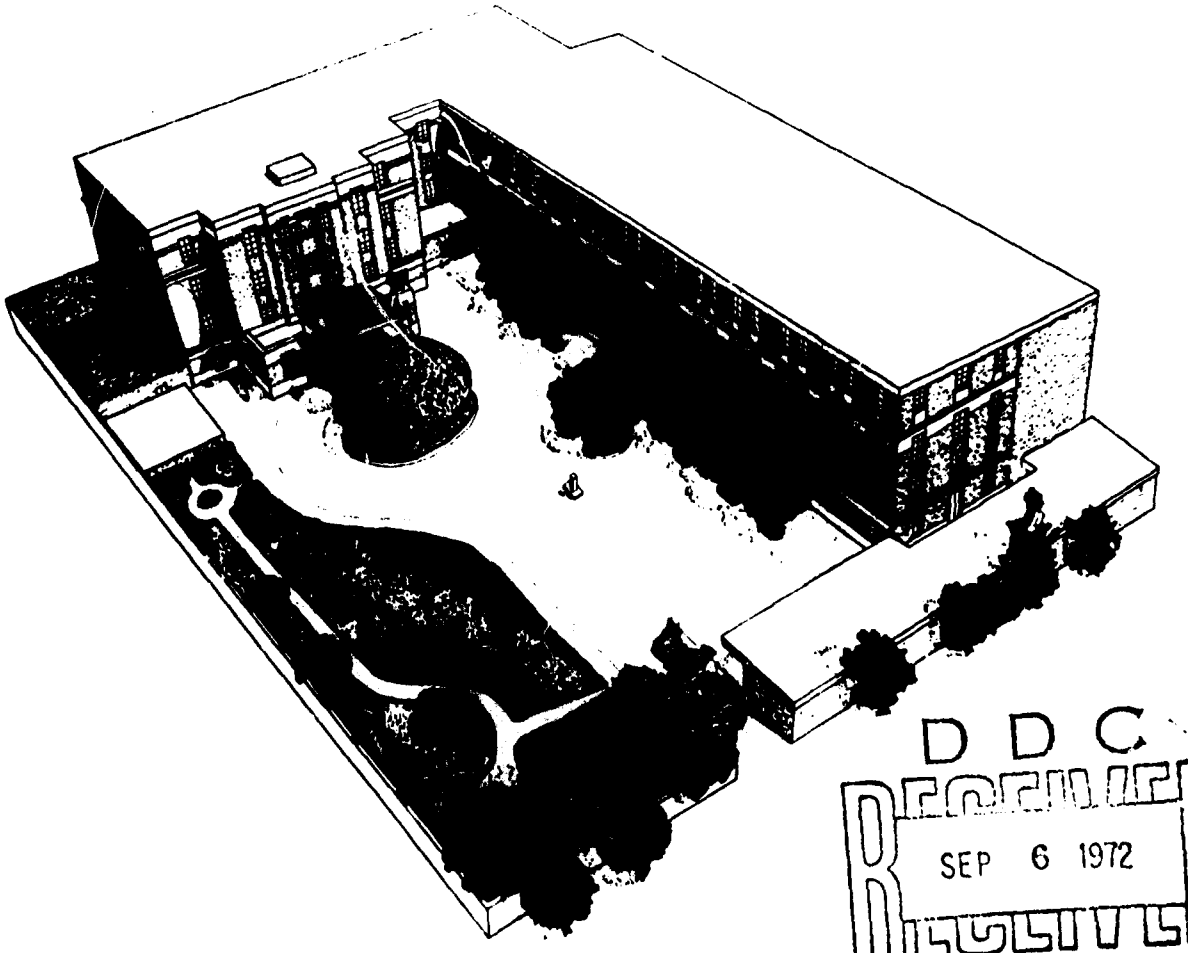
DETECTION OF AN ENDOTOXIN-LIKE SUBSTANCE
DURING HUMAN PL' 'E BACTEREMIA



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13 ABSTRACT

THIS REPORT DESCRIBES A BIOASSAY FOR DETECTION OF ENDOTOXIN ACTIVITY IN STORED PLASMA. THE PROCEDURE IS BASED ON THE ABILITY OF ACTINOMYCIN-D TO ENHANCE SUSCEPTIBILITY OF MICE TO ENDOTOXIN. APPLICATION OF THIS METHOD HAS PERMITTED DETECTION OF AN ENDOTOXIN-LIKE SUBSTANCE IN A PATIENT WITH TERMINAL PLAGUE BACTEREMIA.

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DETECTION OF AN ENDOTOXIN-LIKE SUBSTANCE DURING HUMAN PLAGUE BACTEREMIA

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INTRODUCTION

The causative agent of bubonic plague is *Pasteurella pestis*, a gram negative rod which exhibits bipolar staining with methylene blue stain. Patients dying with this disease display symptoms characteristic of intoxication with endotoxin (Albizo and Surgalla, 1970; Walker, 1967; Walker, 1968). A protein exotoxin which is lethal to rats and mice also has been associated with plague bacilli. Some people believe death during human plague may be caused by this exotoxin alone or by a synergistic action of exotoxin and endotoxin (Walker, 1967).

Although plague exotoxin is toxic to rats and mice when given in small amounts, little or no effect is seen in larger animals (Rust *et al.*, 1963). Furthermore, the amount of exotoxin protein present in the numbers of bacteria required to kill animals is too small to produce death. In contrast, plague endotoxin possesses sufficient toxicity to contribute to or account for death in plague (Albizo and Surgalla, 1970).

The data presented in this report will lend further support to the endotoxin concept of plague toxicity. The author will also describe a modification of the method of Pieroni *et al.*, (1970) to quantitate endotoxin in stored plasma of patients with gram negative bacteremia.

This study was supported through funds provided by the Bureau of Medicine and Surgery, Navy Department, for Work Unit MR041.20.01-0362A.

The research described in this report involved animals maintained in animal care facilities fully accredited by the American Association for Accreditation of Laboratory Animal Care.

The opinions and assertions contained herein are those of the authors and are not to be construed as official or as reflecting the views of the Navy Department or the Naval Service at large.

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MATERIALS AND METHODS

Supplies

Random-bred female white mice weighing 20-25 gm were used for assay of endotoxin. Commercially prepared *Salmonella typhosa* endotoxin extracted either by the Boivin or Westphal procedures was obtained from Difco Laboratories. Actinomycin D (AMD), lot number 3050M, was prepared by Merck Sharp & Dohme, West Point, Pa. Streptomycin sulfate, U.S.P., was prepared by Chas. Pfizer & Co., Inc., New York, N. Y.

Endotoxin Assay

Susceptibility of mice to endotoxin was enhanced (Dowling and Feldman, 1970; Pieroni *et al.*, 1970) by pretreatment with 20 μg AMD injected intraperitoneally 1-4 hours before challenge. Plasma samples were filtered through a Swinney adapter and stored at −10 C. Undiluted plasma and serial dilutions of this plasma in 4.5 ml saline were treated with streptomycin to protect against infection (0.14 mg/mouse). Each sample so treated was injected intraperitoneally into 4 mice (0.6 ml/mouse).

Endotoxin quantitation

The method of Reed and Muench was used to determine the 48-hour LD_{50} of injected samples. Quantitative endotoxin content of samples was obtained by comparison of these

results with those obtained by inoculation of mice with known concentrations of *S. typhosa* endotoxin extracted by the Westphal method.

Animal plasma preparation

Male guinea pigs weighing 300-350 gm were inoculated intraperitoneally with 5 ml of 10^9 viable cells of *Staphylococcus aureus* or *Klebsiella pneumoniae*. These bacteria were maintained on BHI slants and then cultured overnight in BHI broth. Prior to inoculation these bacteria were washed three times in saline. As guinea pigs became moribund (approximately 5 hours) blood was obtained by cardiac puncture. This blood was transferred to heparinized tubes and an aliquot taken for a viable count. Plasma from guinea pig blood was treated in the manner described above.

Patient

Blood was obtained from a patient admitted to the DaNang Medical Center with suspected bubonic plague during March 1971. The patient was a 35-year-old female admitted with a 3-day history of fever, chills, and vomiting and one-day history of groin pain. The temperature was 37.8 C, pulse 170/min, and blood pressure 104/84. She was obtunded, was hyperventilating, and the distal extremities were cool. An ECG showed sinus tachycardia, low voltage, and ST-segment depression. The blood smear showed many bipolar bacilli among the red cells. Antibiotics and intravenous fluids were administered but she died two hours after admission.

RESULTS

Sensitivity of the bioassay was determined by comparison of known amounts of endotoxin obtained either by the Westphal or the Boivin procedure as shown in Table 1. These data are very similar to those obtained by Dowling and Feldman (1970). The Westphal extraction is the more toxic of the two and for this reason was chosen as the standard for subsequent experiments with plasma containing unknown amounts of endotoxin. The amount of endotoxin in blood required to produce clinical effects is actually unknown (Levin *et al.*, 1970). Data obtained in this investigation indicate that quantities in the range of 0.1 µg can be readily detected. Use of other mouse strains or AMD lots may increase this sensitivity (Pieroni *et al.*, 1970).

Table 1

Per cent mortality* of AMD-treated mice challenged with endotoxin.

Conc.	*S. typhosa* endotoxin		Dil.	Guinea pig plasma			Human plasma (Plague patient)
	Westphal	Boivin		Normal	*S. aureus*†	*K. pneumoniae*‡	
10 µg/ml	100	80	0	0	0	86	100
1.0	88	50	10^1	-	-	57	100
0.1	43	30	10^2	-	-	13	60
0.01	11	0	10^3	-	-	0	14
0.001	0	0	10^4	-	-	0	10
LD_{50}(µg/ml)	0.14	1.00	Sample LD_{50} conc. µg/ml§	-	-	0.07 2	0.01 14

* At 48 hours postchallenge, based on 4 mice challenged with each dilution.
† $3.6 \cdot 10^4$ viable organisms per ml guinea pig blood, 5 guinea pigs tested.
‡ $6.0 \cdot 10^7$ viable organisms per ml guinea pig blood, 5 guinea pigs tested.
§ Standard (Westphal) LD_{50}/Sample LD_{50}.

Specificity of the bioassay is indicated by application of the bioassay to plasma from guinea pigs infected with the gram negative organism *K. pneumoniae*. Detectable levels of endotoxin were demonstrated (Table 1). Plasma from uninfected guinea pigs or animals infected with the gram positive organism *S. aureus* exhibited no toxic activity.

Plasma from the patient diagnosed to have bubonic plague was assayed for endotoxin-like activity (Table 1) and was strongly positive.

DISCUSSION

No attempt was made to purify the plasma that was injected into the actinomycin-pretreated mice. Therefore, the mouse-lethal factor could have been an exotoxin or endotoxin, or other toxic substances circulating in the infected plague patient.

Several lines of evidence strongly suggest that the toxic factor may be endotoxin in nature. Actinomycin D appears to be specific in its effect and has not been demonstrated to enhance the toxicity of any other bacterial toxins besides endotoxin (Pieroni *et al.*, 1970). It is hoped that laboratories involved in studies of *P. pestis* exotoxin will verify the specificity of AMD with relation to plague exotoxin and endotoxin.

On the basis of rat studies (Rust *et al.*, 1963), evidence of cardiac toxicity should be observable if exotoxin is present and if human cardiac tissue is susceptible to this toxin. Electrocardiograms of rats injected with sublethal doses of plague exotoxin show specific ST-segment alteration (Rust *et al.*, 1963). ECG studies of the human plague patient, however, gave no clear indication of this phenomenon.

Finally, similarity of death rates of mice challenged with plasma from *K. pneumoniae*-infected guinea pigs and the *P. pestis* patient suggest that the effects reported in this paper are due specifically to the action of endotoxin. Most mice in these groups died between 6 and 24 hours post challenge.

If more plasma had been available from the patient the heat-stability of endotoxin in contrast to the heat-lability of exotoxin would have permitted more definite separation of toxin effects.

Several factors should be considered in the interpretation of the bioassay for endotoxin used in this investigation. Dowling and Feldman (1970) obtained LD_{50} data at 48 hours and 7 days. These investigators, however, used much smaller doses of AMD. In this study deaths from endotoxin began to occur within 6 hours. At 48 hours one of the 10 control mice had died and mice dying thereafter did so in a random fashion. Autopsies of dead or moribund mice after 48 hours indicated that these deaths were due to secondary infections probably as a result of lowered resistance. Heart blood and liver and spleen smears implicated a number of opportunistic pathogens including nonpathogenic Neisseria, *Bacillus subtilus*, Klebsiella, Coliforms, Enterobacter, and *Staphylococcus epidermidis*. Forty-eight hours appear to be the most appropriate time to terminate observations and get maximum sensitivity with minimal contamination.

Quantitation of endotoxin produced during plague by comparison with the Westphal preparation may not be entirely reliable. There is no evidence that the commercial preparation is as toxic as the naturally occurring *Pasteurella pestis* endotoxin. This comparison, however, should be valid in a relative sense.

SUMMARY

This report describes a bioassay for detection of endotoxin activity in stored plasma. The procedure is based on the ability of

actinomycin-D to enhance susceptibility of mice to endotoxin. Application of this method has permitted detection of an endotoxin-like substance in a patient with terminal plague bacteremia.

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Dr. Butler (personal communication) corroborated the presence of endotoxin in the plasma of the plague patient by assaying the sample with the *Limulus* lysate technique.

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